Enrichment and Characterization of Indigenous Anaerobic Ammonium Oxidizing (anammox) Bacteria from Wastewater Treatment Plants in Tshwane, South Africa

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by

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EXECUTIVE SUMMARY

This study set out to investigate the anaerobic ammonium oxidation (anammox) process for removal of nitrogen from wastewater. Removal of the nutrients (N and P) from wastewater is a critical step in the prevention of eutrophication in receiving waters. Biological processes for nutrient removal have been utilised conventionally due to their cost effectiveness compared to the physicochemical processes. The conventional process for nitrogen removal is the nitrification/denitrification process which consists of the oxidation of ammonium to nitrate in two steps:

1) Oxidation of ammonium to nitrite by means of ammonia oxidizing bacteria (AOB), and
2) Oxidation of nitrite to nitrate by means of nitrite oxidizing bacteria (NOB). These two steps are followed by reduction of nitrate to nitrogen gas by the heterotrophic bacteria in the absence of oxygen. However, this process is not suitable to treat high nitrogen loaded wastewaters, i.e. wastewaters characterized by low COD/N ratios.

The latest alternative method to remove nitrogen from nitrogen-rich effluents is the anaerobic ammonium oxidation (anammox) process. This process is carried out by autotrophic bacteria of the type *Planctomycetes* called anammox bacteria which combine ammonium and nitrite to produce nitrogen gas and a small amount of nitrate in anoxic conditions. However, the slow growth of the anammox bacteria in comparison with the heterotrophs makes the start-up and the later development of the anammox process difficult.

Therefore this work was focused on the study of the anammox process. The overall objective of this work was to establish the basis for the implementation of an anammox reactor at lab-scale to remove ammonium from highly-loaded wastewaters.
In order to achieve this, these following specific objectives were done

- To establish the basic method for the enrichment of anammox biomass and to detect and characterize the anammox bacteria.
- To design, develop and optimize the anammox process that can be operated in the most appropriate reactor configuration for the removal of nitrogenous compounds in wastewaters.
- To utilize a molecular methodology for identifying and characterizing anammox bacteria and monitoring the microbial community stability during reactor operation.

These objectives have been developed in the different chapters of the report and the main contents of each chapter of are summarized below:

Chapter 1: A literature review on nitrogen removal from wastewater is discussed. Economic analyses of different technologies for nitrogen removal from wastewater are also included. Additionally, the microbiology and application of the latest innovative technology called the Anammox process is described. Finally, methods for monitoring the stability of the microbial population in an anammox system are also included.

Chapter 2: In this chapter, the enrichment of anammox bacteria is described. The suitable conditions to isolate and develop anammox biomass from WWTP sludge were analysed, for further application of the process. For this purpose, sludge samples from different plants were collected and used to inoculate batch reactors to determine the presence and activity of anammox. After several attempts, anammox biomass was enriched from a sludge samples collected in Daspoort and Zeekoegat municipal WWTP. Anammox activity was detected after 90 days of operation which was detected by simultaneous consumption of NO_2^- and NH_4^+ in the system. This presence of anammox bacteria was also confirmed by the PCR amplification of the 16s rRNA of the anammox bacteria.

Chapter 3: After successful enrichment of anammox in batch reactors, long-term stability of the anammox process using larger volume SBR systems was assessed. The SBR was operated for 120 days and the NLRs of 0.134 g N/L·d and 0.176 g N/L·d were used, respectively. Over the period of operation the nitrogen removal efficiency of 90% was reached with 97% of nitrite consumption. The second part of this chapter was to determine the anammox bacteria in the system and it was found that the anammox bacteria present were closely related to *Candidatus Brocadia anamoxidans*.

Chapter 4: In this chapter the entire microbial population present in the sequencing batch reactor of the anammox system was determined. This was done to better understand the taxonomic diversity present in the reactor. For this reason the next generation sequencing was performed for entire microbial diversity analysis. The system was dominated by bacillus species but a considerable amount of Planctomycetes and anammox was also found.

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Post graduate student

This project was part of the work for a PhD student (Phumza Tikilili) at the University of Pretoria. She is busy with writing her thesis to complete her studies.

Conferences

Tikilili P. V., Chirwa E. M. N. (2014). Searching for Anammox in the South African habitats: Pretoria region. Water Institute of Southern Africa Biennial Conference and Exhibition (WISA 2014), 25-29 May 2014, Mbombela, South Africa.

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LITERATURE REVIEW

1.1 Introduction

Nitrogen compounds are among the most significant pollutants of wastewater because of their role in eutrophication and their toxicity to aquatic life, including human beings (Paredes et al. 2007). Therefore, fixed nitrogen such as ammonium (NH_4^+) and nitrate (NO_3^-) must be removed to avoid toxic algal growths in the environment.

Biological nitrification-denitrification process is most commonly used for nitrogen removal from wastewater. In this process complete nitrification means the oxidation of ammonia to nitrite, and then to nitrate, whereas denitrification is the anoxic reduction of nitrate into nitrite, then into nitrous oxide, nitric oxide, and finally into N_2 gas (Zeng et al. 2009). This process for nitrogen removal requires a lot of energy to create aerobic conditions for bacterial nitrification, and also use organic carbon to help remove nitrate by bacterial denitrification.

Recently, a new process was discovered in which ammonium was converted to dinitrogen gas under anaerobic conditions with nitrite as the electron acceptor (Mulder et al. 1995). This anaerobic ammonium oxidation (anammox) process is a promising low-cost alternative to conventional denitrification systems for ammonium removal from concentrated wastewaters (Strous et al. 1997). The anammox process is autotrophic; therefore there is no need for COD addition to support denitrification (Van de Graaf et al. 1996). In addition, when the Anammox process is combined with a preceding nitrification step, only part of the ammonium needs to be nitrified to nitrite while the Anammox process combines the remaining ammonium with this nitrite to yield dinitrogen gas. This reduces oxygen demand in the nitrification reactor and therefore leads to a second reduction in operation costs. The Anammox process has very low biomass yield and as a result little sludge is also produced. The low sludge production is the third factor that adds up to the significantly lower operation costs compared to conventional denitrification systems. All the above mentioned characteristics make the Anammox process an appealing option to remove ammonium from concentrated wastewaters.

1.2 Nitrogen removal in wastewater treatment plants

It is extremely important to remove nitrogen to the maximum amounts possible during wastewater treatment because of its contribution to the eutrophication of receiving waters, and the toxicity and direct threat it poses to aquatic life (Daims, Taylor and Wagner 2006). The nitrogen is mainly present in wastewaters in the form of ammonium (NH_4^+) (Van Hulle et al. 2010) and can be removed by physicochemical

or biological processes (Zhang et al. 2008). Nowadays there are several ways to reduce nitrogen content from wastewater. In this section both the well-established and the innovative treatment technologies for nitrogen removal will be discussed. Biological treatment to remove nitrogen from wastewater is less expensive and more effective than physicochemical treatments and thus has been used more often to achieve nitrogen removal from domestic wastewaters (Khin and Annachhatre 2004). However, in practice the selection of either a biological or a physiochemical method is determined by the nitrogen concentration of the wastewater. Three concentration ranges can be distinguished (Mulder 2003):

a) Diluted wastewater with ammonium concentration up to 100 mg N/ L (e.g. domestic wastewater). In this range biological processes such as activated sludge are preferred processes based on cost-effectiveness.

b) Concentrated wastewater with higher ammonium concentrations ranging from 100-5000 mg N/L (i.e. leachate, reject water from dewatering of sludge, slurry from farms, etc.) (Van Hulle et al. 2010) after extensive investigations, biological treatment (nitrification-denitrification) process over nitrite is used.

c) Concentrated wastewater with ammonium concentrations higher than 5000 mg N/L. In this range physicochemical methods are technically and economically feasible. The main physicochemical processes applied for ammonium removal are air stripping; breakpoint chlorination and selective ion exchange.

The conventional processes for biological removal of nitrogen are sequential nitrification and heterotrophic denitrification.

1.3 Innovative and sustainable technologies for biological nitrogen removal

The conventional biological nitrogen removal processes are generally used for treating wastewaters with quite low nitrogen concentrations (concentration less than 100 mg N/L) (Van Hulle et al. 2010). Some wastewater streams consist of high concentrations of nitrogen, mostly in the form of ammonium. If these streams are returned back to the inlet of the municipal WWTP, they increase the ammonium loading in the mainstream. Conventional biological nitrogen removal process (denitrification-nitrification) is uneconomical and complicated when treating high nitrogen containing wastewaters with low C/N ratio. During the last decade, several new sustainable and cost-effective alternatives have been discovered and studied and their implementation can be a valid option to treat strong nitrogenous wastewaters characterized by high ammonium concentrations and low biodegradable organic matter content. Currently, anammox process is considered to have potential for treating wastewaters with high ammonium concentrations (He et al. 2007, Ma et al. 2011).

1.4 Microbiology of anammox

Ever since the anammox process was discovered in Delft and the first anammox organism, *Candidatus Brocadia Anammoxidans* was identified, many more of studies have reported the presence of anammox in natural environments such as marine sediments (Dalsgaard et al. 2003; Kuypers et al. 2003, Thamdrup and Dalsgaard 2002), marine sponges (Mohamed et al. 2009), estuarine/tidal river sediments (Trimmer, Nicholls and Deflandre 2003; Rysgaard et al. 2004; Meyer, Risgaard-Petersen and Allen 2005; Dale, Tobias and Song 2009), deep-sea hydrothermal vents (Byrne et al. 2008), hot spring (Jaeschke et al. 2009), and some freshwater ecosystems (Schubert et al. 2006; Robert Hamersley et al. 2009; Penton, Devol and Tiedje 2006; Zhang et al. 2007).Thereby, new species other than *Candidatus Brocadia Anammoxidans* were discovered and identified and their 16srRNA were determined. These were *Candidatus Kuenenia stuttgartiensis, Candidatus Scalindua sorokinii, Candidatus Scalindua brodae, Candidatus Scalindua wagneri, Candidatus Brocadia fulgida and Candidatus Anammoxoglobuspropionicus* (Kuypers et al. 2003; Schmid et al. 2000; Schmid et al. 2000; Kartal et al. 2007b; Kartal et al. 2007a). The Anammox organisms are similar to each other in their 16S rRNA sequences phylogenetic analyses (Figure 1-2), which show that they form a monophyletic branch, which consists of five distinct genera with about 90% sequence similarity to each other, within the phylum Planctomycetes. Entirely, all the species within the order of Planctomycetales lack the peptidoglycan, a virtually universal polymer found within the Bacteria domain. Instead, they consist of the protein as the major constituent of their cell walls. This lack of peptidoglycan is a characteristic shared only with the Chlamydiae and the cell-wall-free Mycoplasms within the Bacteria domain, (Lindsay et al. 2001).

Another structural characteristic of Anammox bacteria is the presence of an organelle called anammoxosome (Figure 1.3) which occupies more than 30% of the cell volume (Niftrik et al. 2006). It has been found that the enzyme hydrazine oxidoreductase, which is responsible for the oxidation of the intermediate hydrazine is present exclusively inside the anammoxosome. Furthermore, this compartment is surrounded by a membrane nearly entirely composed of unique ladderane lipids (Niftrik et al. 2006, Damsté et al. 2002).

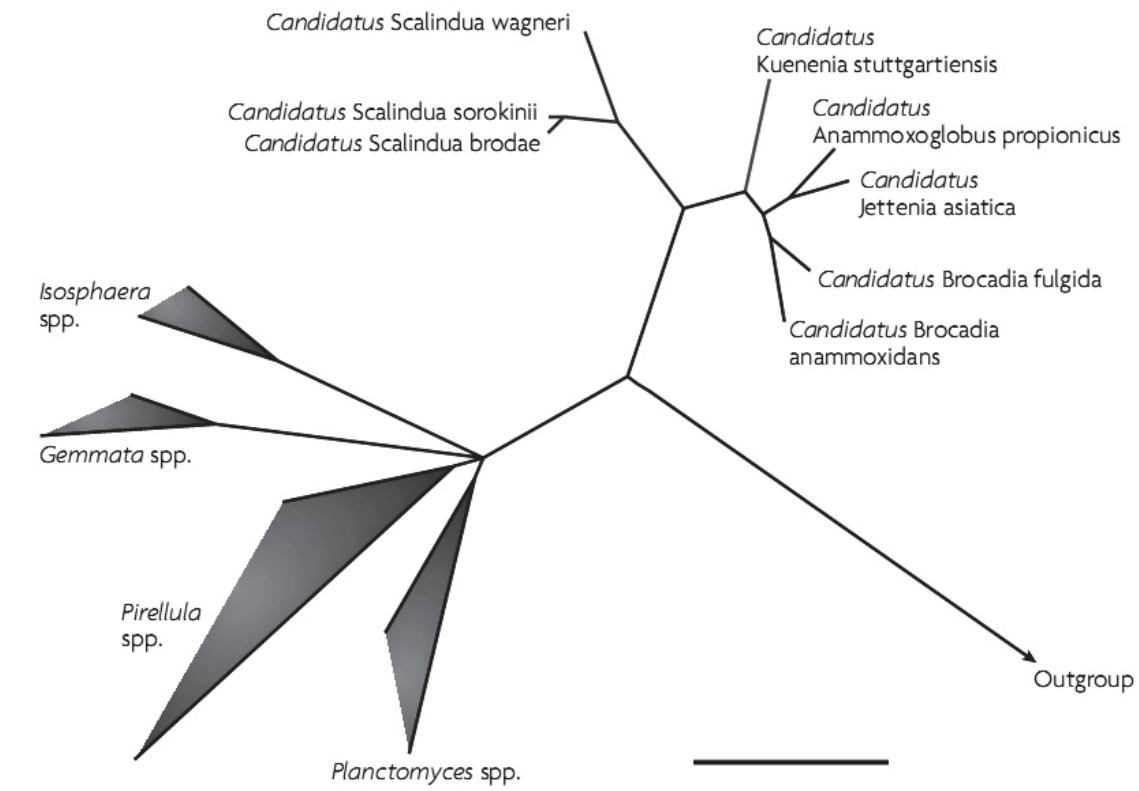

Figure 1-1: 16s rRNA phylogenetic tree of anammox bacteria. The scale bar represents 10% sequence divergence (Source: Kuenen 2008).

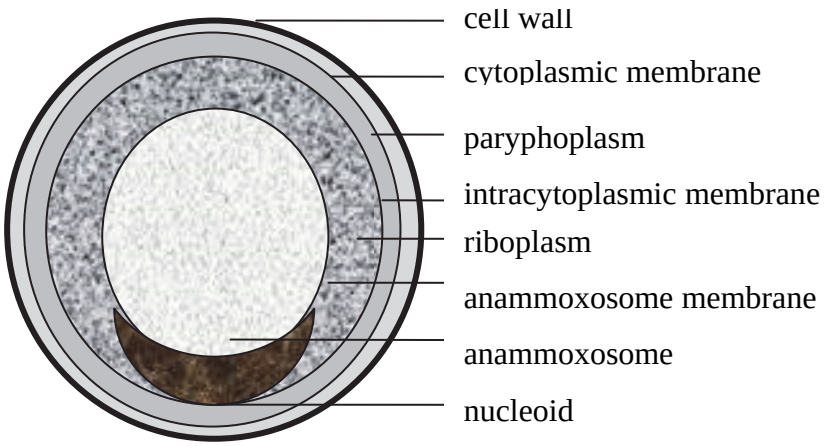

Figure 1-2: Cellular compartmentalization in anammox bacteria (according to *Niftrik* et al. 2006).

1.5 Application of anammox

The discovery and potential contribution of anammox bacteria to N_2 production is important because it may challenge a revisit of nitrogen transformation processes in the global nitrogen cycle (Terada et al. 2011). Research from marine ecosystems suggests that anammox may actually be the major process causing most nitrogen loss in anoxic marine water columns (Dalsgaard et al. 2003; Kuypers et al. 2003). The anammox process has an advantage of low operational costs and has attracted much attention since it was discovered. Anammox is feasible in both natural and man-made ecosystems. In particular, ecosystems with low dissolved oxygen concentrations in surface water and the availability of both ammonium and nitrite/nitrate are considered as prime selectors for the anammox process (Terada et al. 2011). Several studies have been conducted on a variety of ammonium-rich wastewaters (Table1.1). Degradation of organic matter can generate considerable amounts (mM range) of ammonium in natural anoxic environments such as marine sediments (Kuypers et al. 2003; Trimmer, Nicholls and Deflandre 2003). In the case of water columns and marine ecosystems, nitrate reducing bacteria are the most likely source of nitrite (Dalsgaard et al. 2003; Kuypers et al. 2003). Nitrite can also be produced by aerobic ammonium oxidizing bacteria operating at the oxic-anoxic interface of many ecosystems (Schmid et al. 2000). Furthermore, wastewaters from anaerobic treatment of animal waste are also known to contain high organic nitrogen content were also examined for anammox activity (Ahn, Kim 2004; Hwang et al. 2005; Waki et al. 2007). During anaerobic digestion, ammonium is increased considerably mainly due to protein decomposition.

Table 1-1: Anammox application to different wastewaters (source: Zhang et al. 2008).

Wastewater	**Process**	**NRR (kg N/m^3·d)**	**Start-up**	**Scale**	**References**
Sludge liquor	Partial nitritation-anammox	0.71	110	10 L	Van Dongen et al. 2001
Sludge supernatant	Partial nitritation-anammox	2.4	150	2.5 m^3	Fux et al. 2002
Partially nitrified sludge digestate	Anammox	3.5		3.5 L	Fux et al. 2004
Sludge digestate	Partial nitritation-anammox	9.5	1250	70 m^3	Van der Star et al. 2007
Slaughterhouse wastewater	Nitrification-denitrification	0.031		790 mL+745 mL	Reginatto et al. 2005
Piggery wastewater	Anammox	0.6		1.5 L	Ahn and Kim 2004
Piggery wastewater	Partial nitritation-anammox	1.36	~60	1 L	Hwang et al. 2005
Synthetic coke-oven wastewater	Anammox	0.062	~465	1 L	Toh and Ashbolt 2002
Monosodium glutamate wastewater	Anammox	0.46	71	5 L	Chen et al. 2007

1.6 Anammox detection

The discovery of anammox reactions in natural environments and expansion in anammox-related technologies have led to the development of various methods concentrating on detecting anammox bacterial cells/genome (Terada, Zhou & Hosomi 2011). For starting up an anammox process it is necessary to employ the analytical methods that detect the presence of anammox microorganisms even in low concentrations. For this purpose the following methods can be used:

- **Fluorescence In Situ Hybridization (FISH**). FISH relies on DNA/RNA hybridizations occurring within whole microbial cells in situ. In situ hybridizations with DNA oligonucleotides designed for the detection of specific bacteria are performed with fluorescent-labelled compounds. Currently, FISH is well known as a powerful diagnostic tool with widespread environmental and medical applications. It is a fast method, but might not be sensitive enough to detect anammox organisms if they appear in relatively low numbers (below 1000-10,000 cells·mL-1) or if the sample is highly autofluorescent. As more anammox microorganisms are discovered, the designed probes to detect these microorganisms also increase. This permits the detection of more anammox bacteria and the ability to distinguish between the different types (Schmid et al. 2005).

- **The PCR (Polymerase Chain Reaction)**. To detect anammox bacteria in environmental samples, PCR amplification with general 16S rRNA gene-targeted primers and subsequent quantitative or phylogenetic analyses are a central method, in common use. The 16S rRNA or functional gene-based approach without cultivation is a very powerful technique when anammox bacteria have low activity or samples contain a lot of inert particulates, both of which hamper the application of the fluorescence in situ hybridization (FISH) method. It enables the detection of anammox organisms down to the genus level faster and with a higher sensitivity than the FISH method (Schmid et al. 2005).

- **The conversion of hydroxylamine to hydrazine** is a unique reaction of the anammox process (Van de Graaf et al. 1997) that can be used specifically to detect anammox activity in environmental samples (Schmid et al. 2003).

- Much more sensitive are tracer experiments with ^{15}N labeled ammonium. Under anoxic conditions labeled ^{15}N-ammonium reacts uniquely, in a 1:1 ratio with unlabelled ^{14}N-nitrite, to $^{29}N_2$ ($^{14}N^{15}N$) via the anammox reaction. This method was successfully used to assess the contribution of anammox to the nitrogen conversion in marine and estuarine environments, where the cell count of anammox is low (Dalsgaard et al. 2003; Kuypers et al. 2003; Thamdrup, Dalsgaard 2002; Trimmer, Nicholls and Deflandre 2003).

- Anammox bacteria have lipids with unique properties (Damsté et al. 2002; Van Niftrik et al. 2004) that can be used as biomarkers for the presence of these cells in an environmental sample (Kuypers et al. 2003; Schmid et al. 2003).

1.7 Characterization of total microbial population

Bacteria are key players in nutrient removal and comprise a considerable measure of the genetic diversity on Earth (Whitman, Coleman and Wiebe 1998). Different microbial diversity metabolisms such as photoautotrophic and chemoheterotrophic have permitted bacteria to flourish as both free-living and interdependent organisms. Measuring biodiversity includes characterizing the number, composition and variation in taxonomic or functional units over a wide range of biological organizations. There are some difficulties in choosing the suitable unit to measure microbial diversity. Microbial diversity was originally studied through microscopy, and culture on specific liquid and solid media. Performing this type of classification required tedious inspections that relied entirely on morphological characteristics, and bacterial diversity was evaluated mainly by morphotype description of the colony they would form on specific media. Although bacteria are naturally abundant, microbiologists estimated that only 1% of the bacteria counted under the microscope could be cultured on solid or in liquid media, and called this discrepancy the 'Great plate count anomaly'(Staley and Konopka 1985). Therefore, the traditional techniques of isolation and cultivation limit the study of bacteria diversity to those that can be cultivated in laboratory media. Due to the limitations of conventional microbiological methods that are laborious and time consuming (Jasson et al. 2010), different culture-independent methods have been developed to match the conventional microbiology and to acquire a more detailed and precise structure of the total microbial population in the product under survey (Osés et al. 2013).

Various culture-independent molecular methods have been developed to detect many bacterial populations and they provide a more accurate system of taxonomy. The use of molecular techniques in microbial ecology has made it possible for the discovery of several new microorganisms that were previously unknown. Most investigations were mainly based on PCR using specific primers for certain genes in a particular bacterial species. The 16S rRNA is the gene that is normally selected as the target gene because it comprises both variable and conserved regions, permitting the use of primers to conserved regions and more specific primers to amplify16S rRNA genes from any source to distinguish between various taxa (Kawamura, Kamiya 2012). However, since universal primers bind to the same conserved area of the 16S rRNA target population, PCR competitive inhibition can occur, in which the DNA of the prevalent bacterial species is much more likely to be amplified than DNA from bacteria that form a negligible amount of the overall mixed population. This method could be biased towards limited common pathogens (Kuang et al. 2009) and also lead to the complete omission of the DNA of minority bacterial species from analysis (Kawamura and Kamiya 2012). Moreover, the entire bacterial population at a given time cannot be determined by this type of analysis.

Improvements in molecular biology used methods such fingerprinting methods which separate rDNA fragments according to their length and/or their nucleotide composition i.e. PCR–RFLP (restriction fragment length polymorphism), T-RFLP (terminal restriction fragment length polymorphism analysis). Later on, DGGE (denaturing gradient gel electrophoresis) analysis which separates amplified partial 16S rDNA fragments of each bacteria based on differences in the GC content and distribution in each fragment, has been developed and widely used to evaluate the overview of the microbial population in several environmental samples. FISH (fluorescence *in situ* hybridization) approach has also been used. Sanger sequencing method by cloning 16S rRNA gene fragments and subsequently sequencing the clones is also the other method for identifying population diversity. However, cloning/sequencing and FISH are not directly compatible with high-throughput approaches. The most recent and powerful method, metagenomic analysis, has also been applied for the analysis of microbiota. The quest to describe microbial communities has now reached a new stage with the development of next-generation sequencing techniques (NGS), leading towards a high-throughput description of the microbial world at a higher level of detail than cloning or sequencing. This method potentially allows complete analysis of the entire diversity of the microbial population.

1.8 Aim of the project

The main goal of their study was to find out if anammox bacteria exist in some South African anaerobic environments. This project was mainly focused on municipal wastewater to better understand the distribution, diversity, abundance and activity of anammox bacteria in wastewater treatment plants. Therefore, the principal aim of this study was to enrich, isolate and characterize the anammox bacteria from various wastewater treatment plants in the Gauteng region (South Africa).

2. Enrichment of anammox from municipal waste sludge

2.1 Materials and Methods

2.1.1 Wastewater treatment plants Description

A. Daspoort Wastewater Treatment Works (DWWTW)

Daspoort wastewater treatment works consists of two treatment plants i.e. the older "Eastern" Works, and newer "Western" Works respectively. The Eastern Works is a trickling filter (TF) plant that consists of 2 sets of TF systems, Modules 1-4 and 5-6 respectively. On the other hand the Western Works is a conventional biological nutrient removal activated sludge (BNRAS) system which consists of BNRAS systems Modules 9-11. DWWTW receives raw wastewater from the outfall sewer which collects wastewater from the Central Pretoria area. The influent wastewater received by both plants is subject to mechanical screening, grit removal and primary settling in Dortmund-type vertical flow settling tanks.

B. Zeekoegat Wastewater Treatment Works (ZWWTW)

ZWWTW is designed for the capacity of 35 MLD. The raw influent undergoes primary treatment i.e. course and fine screening, grit removal and primary settling in 4 primary settling tanks. This is followed by return activated sludge process with 2 identical biological reactors which are operated as modified UCT process for nutrient removal. The mixed liquor is then diverted to 4 clarifiers. The overflow of the clarifiers is disinfected by chlorine gas before entering filtration unit. The influent is released into the pond to allow for sufficient chlorine contact time. The final effluent is discharged into Lake Roodeplaat via artificial wetland.

C. Baviaanspoort Wastewater Treatment Works (BWWTW)

BWWTW is among the first BNR reactors in South Africa and was constructed in 1977. It has the capacity of 35 MLD. It consists of the primary stage similar to that of Zeekoegat but lacks the division tank. It also consists of four biological nutrient removal activated sludge (BNRAS) modules with a total capacity of 62 MLD. Based on the TKN/COD ratio of the influent, the best suited process configuration was found to be the UCT configuration. However as the raw influent sewage composition differs, the configuration is also changed to a different configuration that best suits the wastewater composition. The reactors consist of the mechanical aeration system. The final effluent is also discharged into Lake Roodeplaat.

2.1.2 Sample collection

Wastewater samples were collected from three wastewater treatment plants in Pretoria namely: Daspoort, Baviaanspoort and Zeekoegat wastewater treatment plants. From Zeekoegat and Baviaanspoort, the sludge samples were collected from both anoxic and anaerobic zones of the activated sludge system. This was done by lowering a sampling jug with an extended handle of about 1-1.5 m in length into the reactor. The sludge samples were

the transferred to 1 L plastic containers. From Daspoort, the sludge samples were taken from an anaerobic digester from the middle (anoxic) and the bottom (anaerobic) part of the digester. This was done by opening the valve in the middle and bottom part of the digester and collecting the samples into plastic containers. In Daspoort the samples were also taken from the trickling filter by taking stones covered with biofilm and were placed in plastic sampling containers. The length from which the stones were taken was not measured but they were only sampled based on the attached growth. All the samples were stored in a room temperature and cultured within 24 hours. The remaining samples were stored at 4ºC for future reference.

2.1.3 Anammox reactor set up and operation

A series of batch reactors were set up and used for cultivation and enrichment of anammox bacteria. Each reactor was a 500 mL serum bottle containing 300 mL of simulated wastewater with the composition adapted from Jetten et al. 2005 (Table 2-1 and 2-2). Each reactor was inoculated with 20% of different activated sludge samples from different wastewater treatment plants. The sludge samples were collected from Daspoort (anaerobic digester), Zeekoegat and Baviaanspoort wastewater treatment plants from anoxic and anaerobic zones of the activated sludge system. Apart from the samples collected from the two mentioned WWTP, trickling filter samples from Daspoort wastewater treatment works were also included in the primary enrichment experiments. To get rid of oxygen from the reactors and create anaerobic conditions, the reactors were purged with argon gas for 15-20 min before sealing with rubber septa and aluminium crimp seals. The reactors were then shaken on a rotary shaker or stirred on a magnetic stirrer at a speed of 80-100 rpm at a temperature of 31° C in the dark controlled room. After 60 days of incubation the synthetic medium was replaced with the freshly prepared one to replace the depleted nutrients. After a total of 90 days of incubation experiment to test anammox activity were conducted in small 500 mL reactors using the biomass from the primary enrichment reactors as the inoculum. 2 mL aliquot sample were withdrawn and analysed duplicate according to the analytical method.

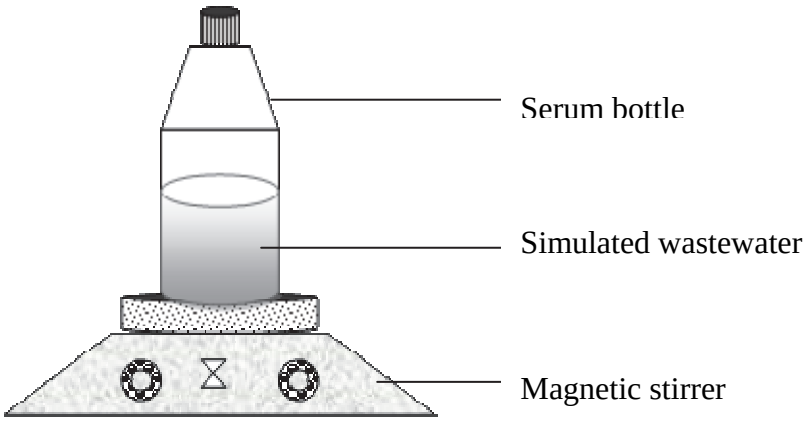

Figure 2-1: Schematic representation of experimental set up

Table 2 -1: Composition of anammox minereal medium

Compound	Concentration (g/L)
KNO_3	0.2
$KHCO_3$	1.25
NaH_2PO_4	0.05
$CaCl_2 \cdot 2H_2O$	0.3
$MgSO_4 \cdot 7H_2O$	0.2
$FeSO_4$	0.006
EDTA	0.006
$NaNO_2$	0.12
$(NH_4)SO_4$	0.15

2.1.4 Analytical methods

Ammonium, nitrite and nitrate were analysed calorimetrically according to the following methods:

- Nitrate analysis – 10 µL saturated sulphuric acid was added to 40 µL reactor effluent together. To the mixture, a total of 0.2 mL reagent containing 5% salicylic acid in 98% sulphuric acid and 2 mL 4 M NaOH (4ºC) was added. This solution was analysed in a Spectrophotometer (Model, company) at 420 nm after a 30 min reaction.
- Ammonium analysis – 760 µL of a solution containing 0.54% ortho-pthalaldehyde, 0.05% β-mercaptoethanol and 10% ethanol in 400 mM potassium phosphate buffer (pH 7.3) was added to 40 µL reactor effluent sample. This solution was analysed in a Spectrophotometer (Model, company) at 420 nm after a 30 min reaction.
- Nitrite analysis – 950 µL of a reagent containing 1% sulfanilic acid and 0.05% N-naphthylethylenediamine in 1 M H_3PO_4 was added to 50 µL of reactor effluent. This is followed by a Spectrophotometric analysis at 540 nm after 5 min reaction.

Table 2-2: Composition of trace elements solution

Compound	Concentration (g/L)
H_3BO_3	0.5
$CuSO_4.5H_2O$	0.04
KI	0.1
$FeCl_3.6H_2O$	0.2
$MnSO_4 \cdot H_2O$	0.4
$Na_2MoO_4.H_2O$	0.2
$ZnSO_4.7H_2O$	0.4
NaCl	1
$CoSO_4$	0.1
$CaCl_2$	0.1
$AlK(SO_4)_2.12H_2O$	0.01

2.1.5 Detection of anammox by 16s rRNA gene sequencing

To detect the presence of anammox in the reactors, PCR amplification targeting the 16S rRNA of the anammox was performed. From the reactors, samples were withdrawn and centrifuged at 6000 rpm for 10minutes. Pellets were re-suspended in phosphate buffered saline (PBS pH 7.4) and centrifuged again. DNA was directly extracted from these samples using a ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) according to the manufactures instructions. The PCR primers used in this study are primer sets AMX 368F – AMX 820R and AMX 368F – BS 820R (Table 3).

Table 2-3: PCR primers for anammox bacteria related 16s rRNA

Primer	Target	Sequence (5' - 3')
AMX 368F	All anammox bacteria except for *Anammoxoglobus propionicus*	TTCGCAATGCCCGAAAGG*
AMX 820R	*Brocadia anammoxidans*, *Brocadia fulgida* and *Kuenenia stuttgartiensis*	AAAACCCCTCTACTTAGTGCCC*
BS 820R	*Sculindua wagneri* and *Scalindua sorokinii*	TAATTCCCTCTACTTAGTGCCC*

* Amano et al. 2007

The PCR reaction mixture (50µl) contained 25 µL of DreamTaq™ Green PCR Master Mix (2X) (Fermentas life sciences), 0.1 µM of each reverse and forward primer, 1 µg of DNA template and nuclease free water. DreamTaq™ Green PCR Master Mix is a ready-to use solution containing DreamTaq™ DNA polymerase, optimized DreamTaq™ Green buffer, $MgCl_2$ and dNTPs. The thermal cycle program used was an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 2 min, final extension at 72°C for 10 min and hold at 4°C. The PCR products were recovered and purified using ExoSAP amplicon purification kit (Inqaba Biotech) according to manufacturer's instructions. Purified DNA fragment were ligated to pJET1.2/blunt cloning vector (Thermo Scientific) and 16s rRNA gene clone libraries were constructed by transforming Premade Z-Competent TM *E. coli* Cells (Zymo Research) with a vector.

Sequencing was carried out with the ABI V3.1 Big Dye kit. Cleaned sequencing products were analysed on the ABI 3500XL genetic analyser (Applied Biosciences, USA) using a 50 cm array and POP7. The phylogenetic analysis was done using the NCBI-BLAST search.

2.2 Results and Discussions

2.2.1 Enrichment of anammox biomass

As it was unknown whether the inoculum contained anammox microorganisms or not and because of the low growing rate and yield of these microorganisms, long enrichment times were employed in this study in order to have detectable quantities of anammox cells by PCR,

if anammox bacteria were initially present in the inoculum. The results that are reported are those obtained from all three wastewater treatment plants. The results obtained from calorimetric analysis of ammonia, nitrite and nitrate are present below (Figures 2-2 to 2-9).

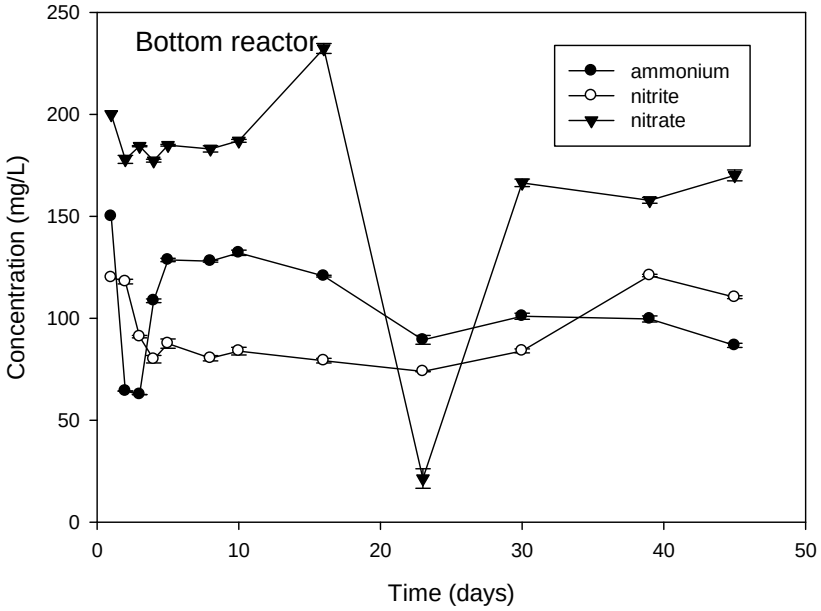

Figure 2-2: Results for Daspoort bottom sample **(**Bottom reactor represents the results for the reactor inoculated with Daspoort sample collected from the bottom part of an anaerobic digester).

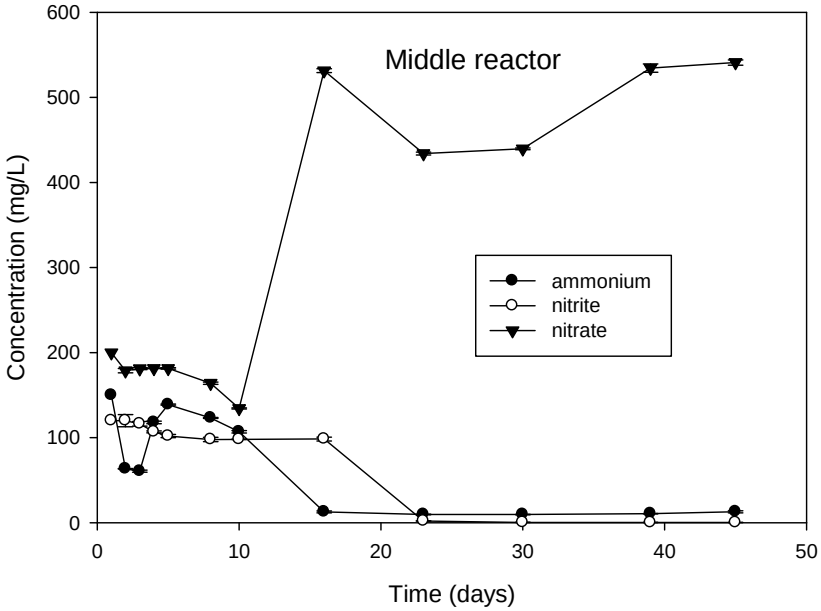

Figure 2-3: Results for Daspoort middle sample (middle reactor represents the results for the reactor inoculated with Daspoort sample collected from the middle part of an anaerobic digester.)

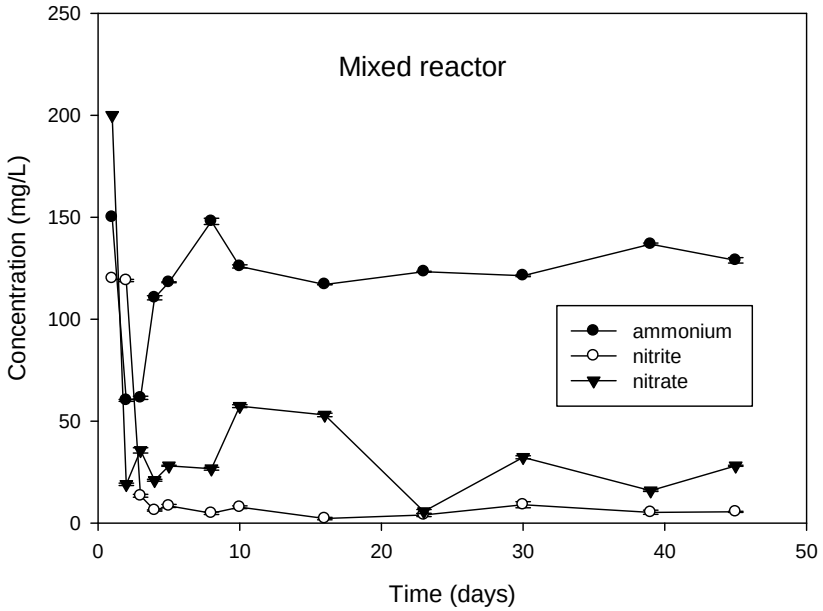

Figure 2-4: Results for Daspoort mixed sample **(**mixed reactor represents the results for mixed Daspoort samples (middle and bottom) enriched together.

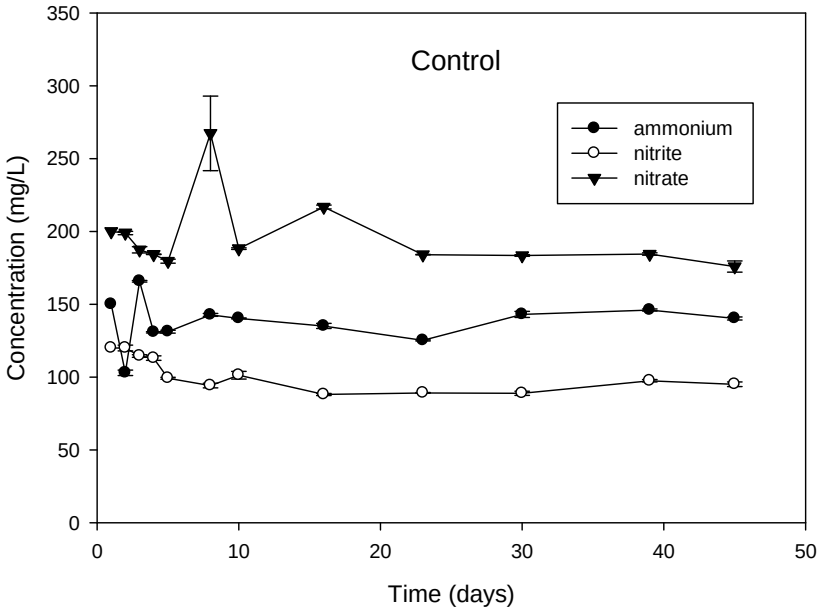

Figure 2-5: Results for the control (control reactor represents the results for the reactor that was not inoculated with sludge).

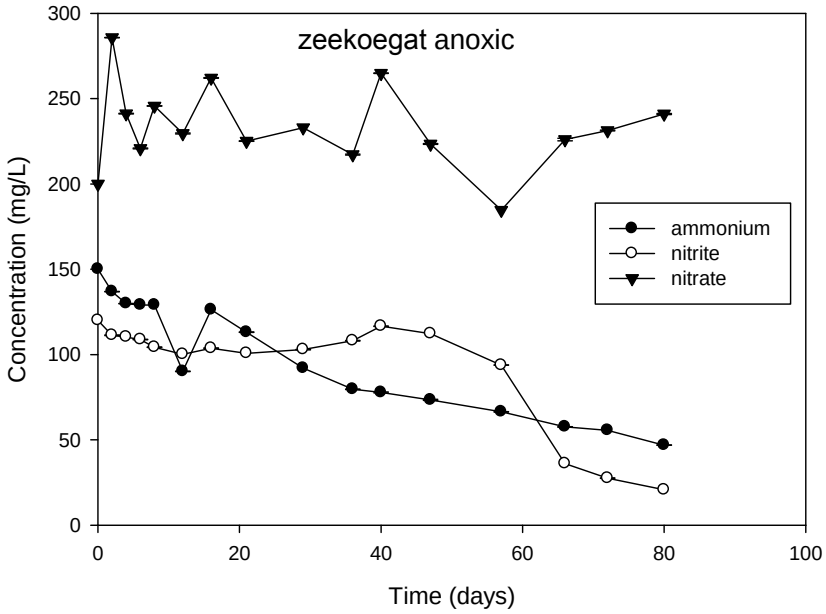

Figure 2-6: Zeekoegat anoxic represents results from reactor inoculated with sludge samples obtained from the anoxic zone of the secondary stage of Zeekoegat treatment plant.

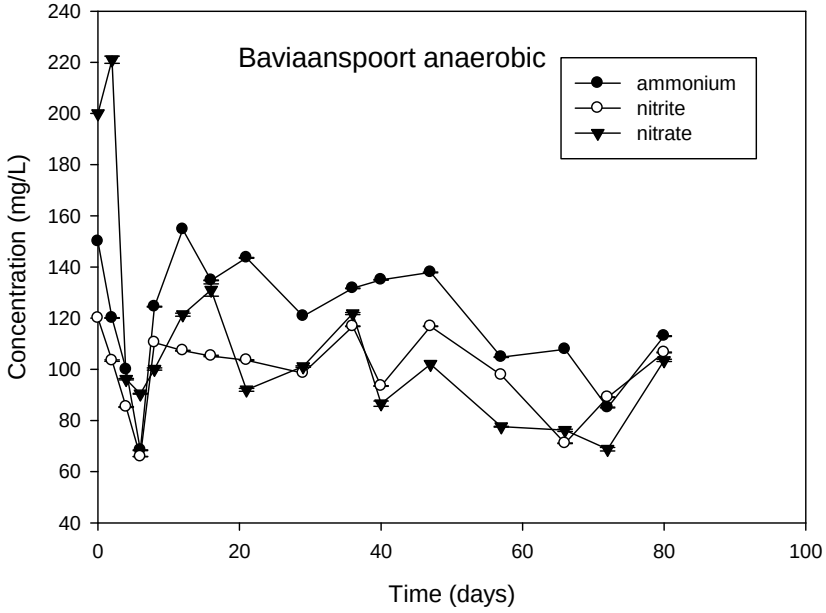

Figure 2-7: Baviaanspoort anaerobic represents results from reactor inoculated with sludge samples obtained from the anaerobic zone of the secondary stage of Baviaanspoort treatment plant.

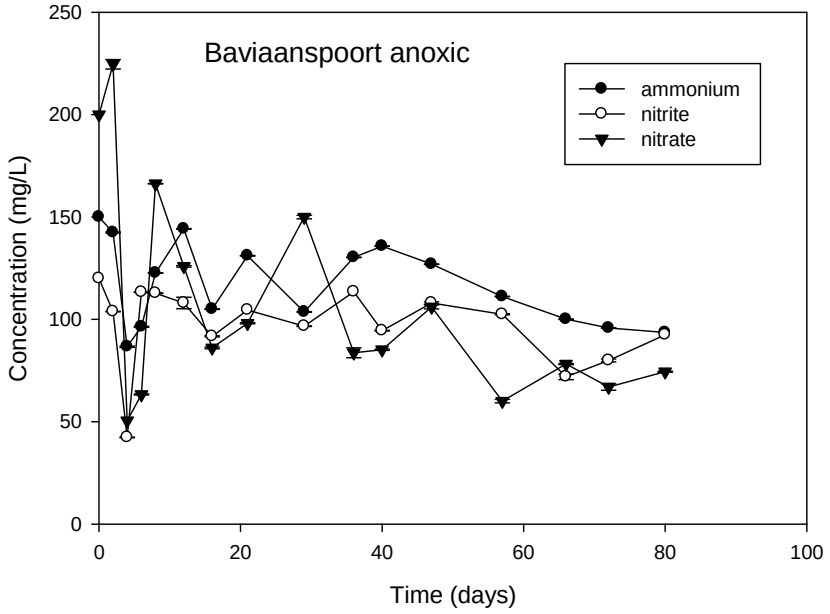

Figure 2-8: Baviaanspoort anoxic represents results from reactor inoculated with sludge samples obtained from the anoxic zone of the secondary stage of Baviaanspoort treatment plant.

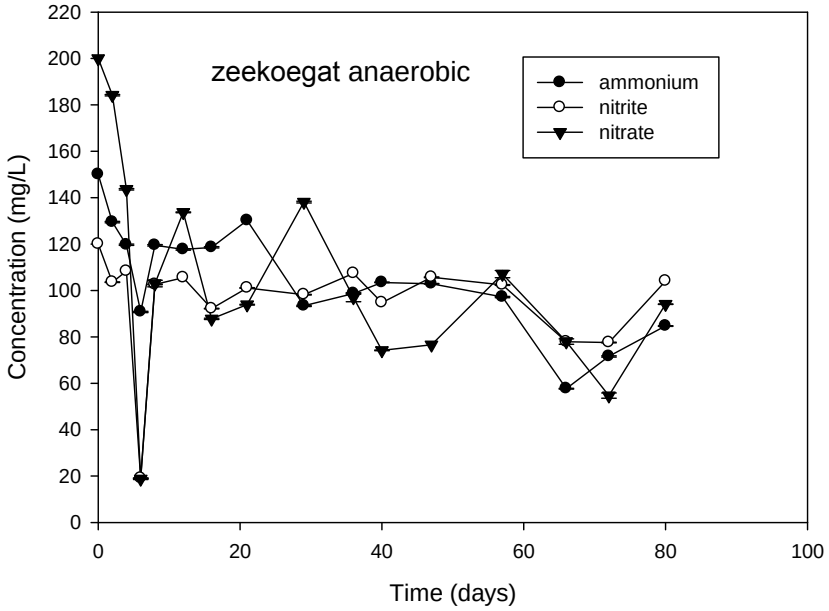

Figure 2-9: Results for sample from Zeekoegat anaerobic zone **(**Zeekoegat anaerobic represents results from reactor inoculated with sludge samples obtained from the anaerobic zone of the secondary stage of Zeekoegat treatment plant).

The Figures 2-2 to 2-4 illustrate the results obtained after primary enrichment of Daspoort samples. Firstly, simultaneous ammonium and nitrite removal were observed in the middle reactor (Figure 2-3). Additionally, an increased production of nitrate was also observed in

the same reactor. All these are characteristics of the anammox reaction clearly demonstrating that an anammox reaction occurred in this reactor.

On the other hand, no considerable consumption of ammonia and nitrite was observed in the bottom and mixed reactors (Figure 2-1 and 2-4) after several days of incubation, suggesting the absence of anammox activity in these reactors. There was no significant change in the control (Figure 2-5) where there were no cells added.

The enrichment of anammox cultures for samples obtained from two other municipal wastewater treatment plants (Zeekoegat and Baviaanspoort) after 90 days of primary enrichment, are presented from Figure 2-6 to 2-9. The anammox activity was tested and confirmed by calorimetric analysis of ammonium, nitrite and nitrate in the batch reactors. Figure 2-6 is showing the results with successful enrichment. This was confirmed by noticeable consumption of ammonium and nitrite which was followed by a slight increase in the nitrate concentrations.

On the other hand, the rest of other experiments were unsuccessful in anammox enrichment. No simultaneous consumption of nitrite and ammonium was observed in these experiments after the duration of incubation. These results are presented in Figures 2-7 to 2-9. Successful enrichment could also be identified by visual observation through colour change of the biomass. Anammox bacteria are characterized by reddish brown colour which was seen in reactors with successful enrichment (Figure 2-10). There was no colour change observed in the reactors with unsuccessful enrichment. The reactor contents remained black like the colour of the sludge (Figure 2-11)

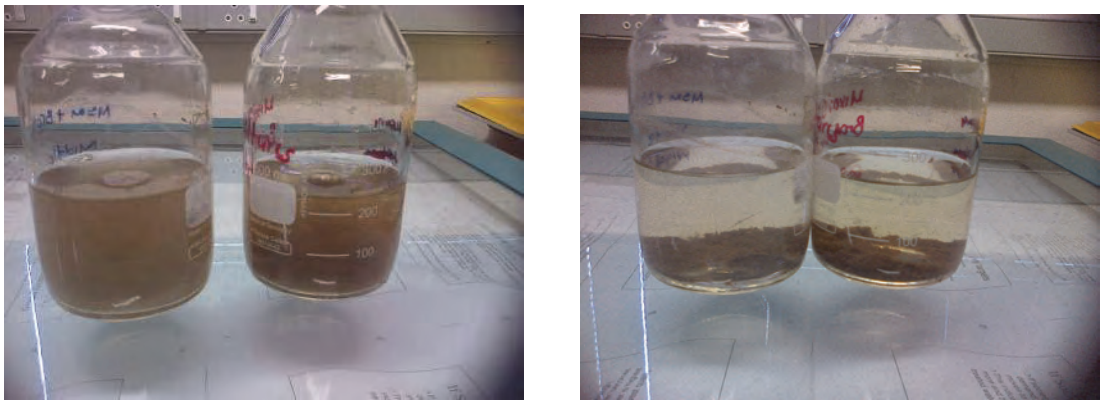

Figure 2-10: Visual observation of cultures with successful enrichment

Figure 2-11: Visual observation of cultures with unsuccessful enrichment

Following these experiments, PCR of DNA extracted from these reactors was performed using anammox specific primers for the detection of anammox in the reactors. In addition to the reactors that were tested in this set of experiments, trickling filter samples were also tested for the presence of anammox. These samples were not included when Daspoort samples were evaluated because at the time of experiments, there was no visible growth of biomass from the trickling filter enrichment reactor. Therefore further incubation was carried out to obtain enough biomass for evaluation.

After a successful enrichment was obtained, PCR using anammox-specific primers Amx368F-Amx820R and Amx368F- BS820R was performed. The PCR for the middle reactor DNA extract resulted in positive amplicons for both primer sets (Figure 2-12). On the other hand no positive bands were observed for both bottom and mixed reactors.

PCR results are presented in Figure 2-13 and are confirming the presence of anammox bacteria in the reactor inoculated with Zeekoegat anoxic zone sludge samples. In addition to these results is another positive identification of anammox bacteria in a trickling filter samples. It was characterized by a bright positive band indicating a high concentration of anammox DNA. The results of these enrichment experiments are summarized below (Table 2-4)

Sequencing of the cloned 16s rRNA fragments resulted in the detection of sequences which were highly similar to known anammox bacteria. The sequences found were identified as *Candidatus Brocadia,* Uncultured *Planctomycetales bacterium* clone Amx16S-ZJ-W-1-9 16S ribosomal RNA gene and uncultured anaerobic ammonium oxidizing bacterium cloneW1 16S ribosomal RNA gene.

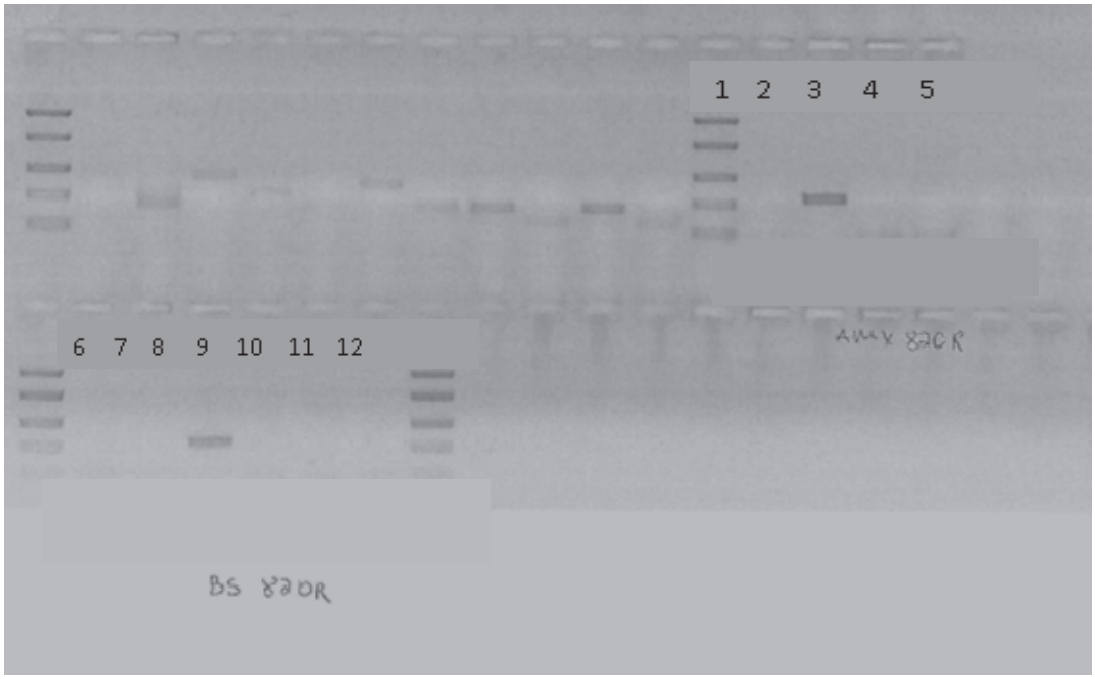

Figure 2-12: PCR products amplified by anmmox specific primers primer sets Amx368F-Amx820R (lane 1-5) and Amx368F-BS820R (lane 6 – 12). Ne - (lane 7and 12) – are negative controls, BR (lane 2 and 8) are bottom reactor, MR (lane 3 and 9) - middle reactor, B+M (lane 4 and 10) - bottom and middle reactors mixed after enrichment, MX (lane 5 and 11) – mixed samples and enriched together.

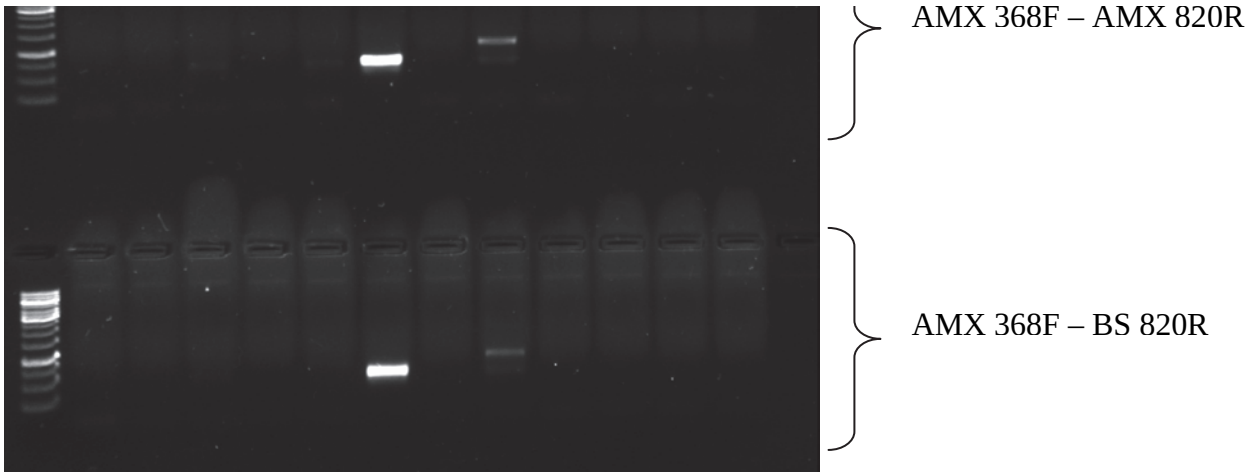

Figure 2-13: PCR products amplified by anammox specific primers primer sets Amx368F-Amx820R (Top lanes) and Amx368F-BS820R (bottom lane). (1) Marker, (2) negative controls (4) Baviaanspoort anoxic zone, (5) Zeekoegat anaerobic zone, (6) Baviaanspoort anaerobic zone, (7) Trickling filter (9) Zeekoegat anoxic zone, (10) negative control

Table 2-4: Summary of Enrichment experiments

WWTP	Zone of Sampling	Enrichment
Daspoort	Trickling Filter	Yes
Daspoort	Anaerobic digester(middle)	Yes
Daspoort	Anaerobic digester(bottom)	No
Baviaanspoort	Anoxic	No
Baviaanspoort	Anaerobic digester	No
Zeekoegat	Anoxic	Yes
Zeekoegat	Anaerobic digester	No

2.3 Conclusion

The results obtained from these experiments showed the presence of anammox from some of the Gauteng wastewater treatment plants. The results also showed the high concentrations of anammox bacteria in Daspoort trickling filter which was shown by the bright positive band of PRC results. The successful enrichment biomass was be used as an inoculum for the following Sequencing batch reactor experiments.

3. Performance of Sequencing Batch Reactor

3.1 Materials and methods

3.1.1 Reactor description

A sequencing batch reactor (SBR) with a working volume of 5 L was used (Figure 3-1). The pH was maintained between 7.5 and 8 without specific control. The medium was homogenized by a magnetic stirrer. A set of two peristaltic pumps was used to introduce the feeding solution and to discharge the effluent. Timers controlled the actuations of the pumps and valves and regulated the different periods of the operational cycle. The reactor was flushed continuously Argon to maintain anaerobic conditions. All the tubing was norprene tubes, to prevent the diffusion of oxygen inside the system

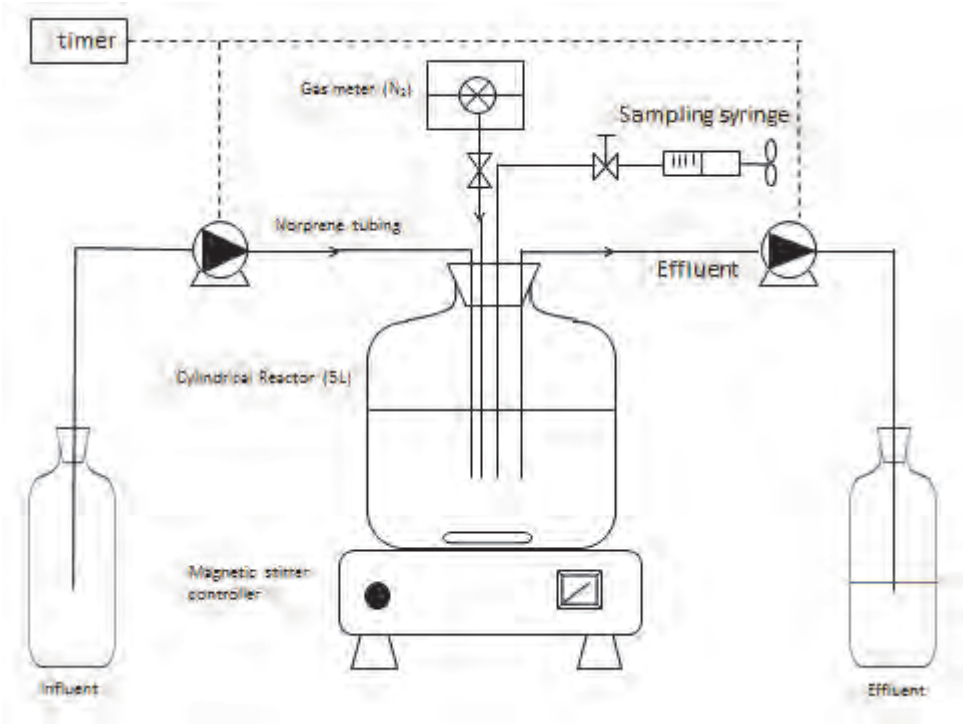

Figure 3-1: Experimental set up of SBR

3.1.2 Inoculum

The reactor was inoculated with a mixture of settled biomass of anammox bacteria from primary enrichment batch reactors.

3.1.3 Operational conditions

The SBR was operated in 12 h cycles distributed as follows during operation: 600 min of feeding and mixing, 45 min of settling, 15 min of effluent withdrawal. The feeding supplied to the reactor was prepared using the mineral salt medium. The reactor was operated in 2

different stages depending on the wastewater composition (Table 3.1.). The hydraulic retention time (HRT) was maintained at 0.4 d.

Table 3-1: The composition of mineral salt medium used as the influent in the SBR

Compound	**Concentration (g/L)**
KNO_3	0.06
$KHCO_3$	1.25
NaH_2PO_4	0.05
$CaCl_2 \cdot 2H_2O$	0.3
$MgSO_4 \cdot 7H_2O$	0.2
$FeSO_4$	0.006
EDTA	0.006
$NaNO_2$	0.06
$(NH_4)SO_4$	0.12*
Trace elements solution	1.25 mL/L

* Increased to 0.15 g/L on day 40 of operation

Table 3-2: SBR operation Conditions

Feeding + mixing			
Settling			
Draw			
Time (minute)	600	65	20

3.2 Results and Discussion

The Sequencing batch reactor for an anammox process was started-up with nitrogen loading rate (NLR) of 0.134 g N/L·d. The reactor was operated for about 120 days. The reactor was operated in two stages. In the second stage, the initial NLR was increased from 0.134 to 0.176 g N/L·d by increasing the concentrations of nitrogen compounds. However, only NH_4^+ concentrations were increased. Nitrite concentrations remained the same as in the first to avoid the inhibitory effect of nitrite in the system. Over the period of 120 days the nitrogen removal efficiency was about 90% with nitrite almost completely consumed (>97%) (Figure 3-2).

When NLR was increased to 0.176 g $N \cdot L^{-1} \cdot d^{-1}$ concentrations of NH_4^+were slightly increased in the effluent and later they started to decrease gradually

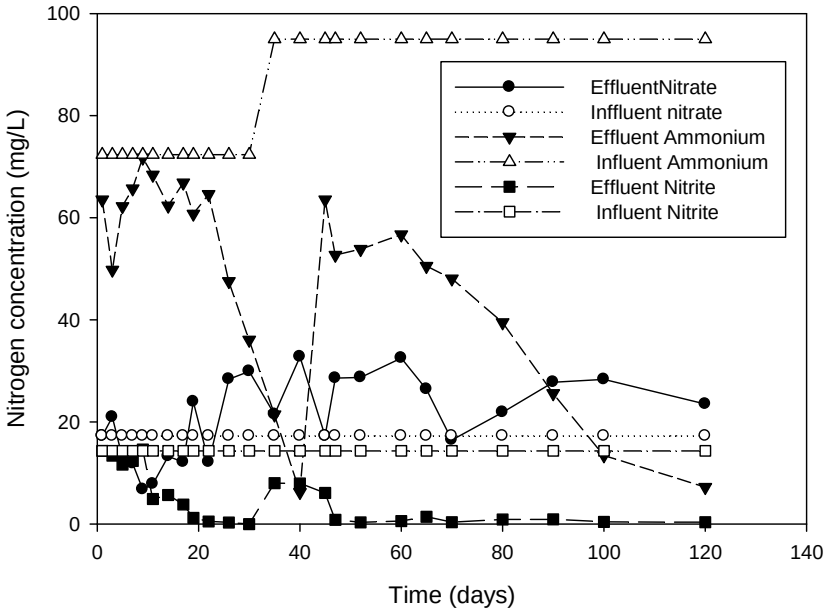

Figure 3-2: Operation of SBR Influent: NH_4^+ Δ, NO_2^- □, NO_3^- ○, Effluent NH_4^+ ▼, NO_2^- ■, NO_3^- ●

In an anammox process nitrogen gas has to be produced. In order to reach the conclusion that anammox was taking place in the reactor, gas production was also monitored. The production of the gas in the reactor was confirmed by a gradual increase in readings of the gas metre that was used. In addition, calculations of nitrogen balance were made and it was found that 1.22 moles of nitrite were consumed and 0.2 moles of nitrate were produced. Though these values are not exactly the same as those of the anammox process, they are close and comparable to the theoretical stoichiometry of anammox. Therefore it can be concluded that anammox process definitely took place in the reactor. To further associate the detection of anammox activity to the presence of anammox bacteria in the reactor, DNA extraction and sequencing was performed on biomass samples that were taken from the reactor. The phylogenetic tree obtained from the sequencing is presented in Figure 3-3. The results showed that the anammox bacteria in the reactor were closely related to *Brocadia* species (*fulgida* and *anammoxidans*). These findings are in agreement with those obtained by Wilsenach et al. (2014) where they detected the same species from the samples collected from Daspoort trickling filters.

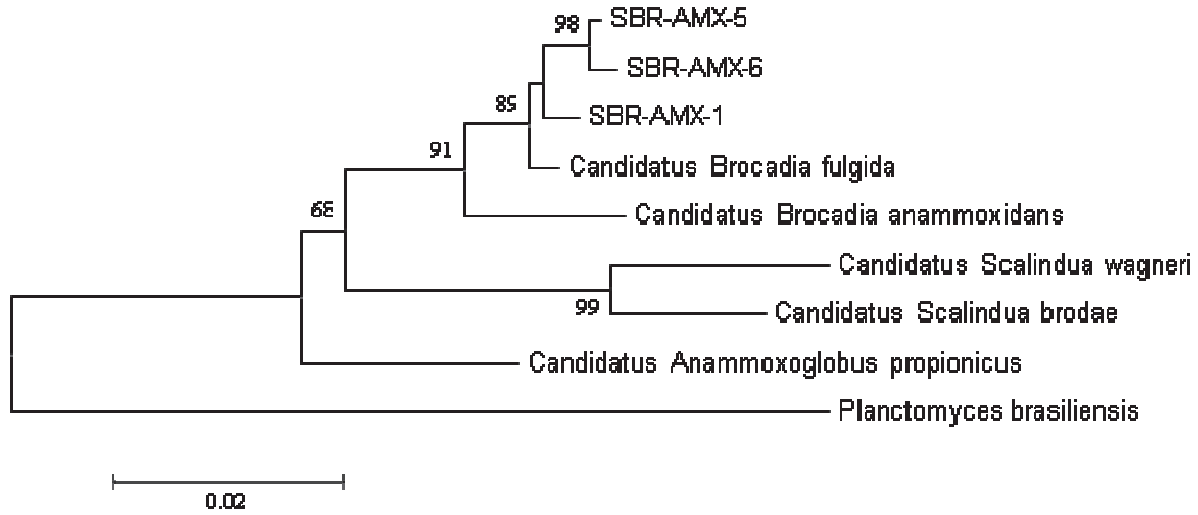

Figure 3-3: Phylogenetic tree showing the relationships of the 16S rRNA gene from the SBR to known anammox species

4. Characterization of Microbial Populations in a Sequencing Batch Reactor

4.1 Objective

The aim of this study was to determine the entire microbial population present in the sequencing batch reactor of the anammox system. The structure and microbial community was determined by the next generation sequencing (NGS).

4.2 Materials and Methods

4.2.1 DNA Sample isolation

Genomic DNAs was extracted from the Sequencing Batch Reactor (SBR). 2 mL Samples were withdrawn from the SBR and centrifuged at 6000 rpm for 10minutes. Pellets were re-suspended in phosphate buffered saline (PBS pH 7.4) and centrifuged again. DNA was directly extracted from these samples using a ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) according to the manufactures instructions.

4.2.2 Paired End library Preparation

Extracted genomic DNA was used to perform PCR to amplify V3 and V4 regions of the 16s rRNA. The gene specific primers used were:

PCR forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'
PCR reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

These primers were selected from (Klindworth et al. 2013). Attached to these primers were overhang adapter sequences with the following primers:

Forward overhang: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus specific sequence]
Reverse overhang: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus specific sequence]

The PCR reaction mixture (25 µL) contained 2.5 µL of genomic DNA (5 ng/µL), 5 µL of each forward and reverse primer (1 µM) and 12.5 µL of 2x KAPA Hifi Hot start Ready mix. The thermal cycle program used was an initial denaturation at 95°C for 30 sec, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 min and hold at 4°C. The PCR was followed by PCR clean up to purify the 16s V3 and V4 amplicons by removing primer dimers and free primers using AMPure XP beads. The clean-up was followed by Index PCR which attached dual indices and Illumina sequencing adapters using Nextera XT Index kit. The PCR reaction (50 µL) was composed of 5 µL DNA 5 µL of each Nextera XT index primer 1(N7xx) and 2(S5xx), 25 µL of 2x KAPA Hifi Hot start Ready mix, 10 µL PCR grade water. The PCR conditions used were an initial denaturation at 95°C for 3 min, followed by 8 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72 °C for 30 sec,

final extension at 72°C for 5 min and hold at 4°C. Second PCR clean-up was performed using AMPure XP beads. The final purified DNA was the quantified using fluorometric quantification methods.

4.2.3 Sequencing

The sample containing the indexed amplicons was loaded on the miseq reagent cartridge, and then onto the instrument along with the flow cell. Automated cluster generation together with paired end sequencing was performed. Total run for this 2 x 300 bp was 40 h.

4.3 Results and Discussions

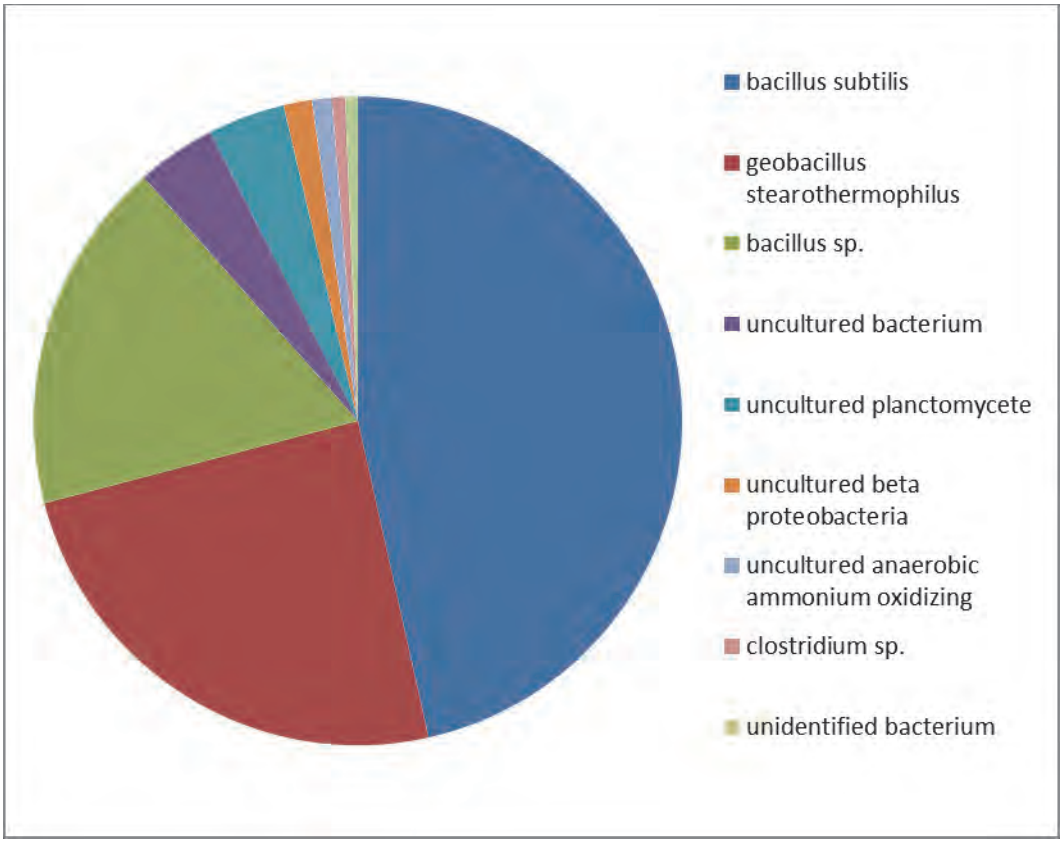

Figure 4-1: Dominant bacterial species in SBR

In order to identify the main microorganisms forming part of the microbial population, metagenomics using next generation sequencing was performed. The main objective was to determine how much of anammox bacteria are in the system and what other organisms are present. The sample for this analysis was taken towards the end of reactor operation in order to determine the true microbial community during the operation of the reactor. After data analysis using MiSeq Reporter (MSR), results presented in Figure 4-1 were obtained.

Table 4-1: Diversity of microbial population in SBR

Bacterial species	**Cluster count**	**Percentage**
Bacillus subtilis	52192	44.18707034
Geobacillus stearothermophilus	27396	23.19414813
Bacillus sp.	19769	16.73693657
Uncultured *Bacterium*	4412	3.73531105
Uncultured *Planctomycete*	4331	3.666734397
Uncultured *Beta Proteobacteria*	1579	1.33682143
Uncultured anammox	1116	0.944833892
clostridium sp.	730	0.618036506
Unidentified *Bacterium*	709	0.600257374
Uncultured *Nitrospira*	680	0.575705239
Burkholderia pseudomallei	511	0.432625555
uncultured soil	487	0.412306546
Nocardia sp.	431	0.364895526
Bifidobacterium sp.	389	0.329337262
Pseudomonas aeruginosa	366	0.309864879
Achromobacter xylosoxidans	259	0.219275966
Agrobacterium tumefaciens	251	0.212502963
Pseudoxanthomonas sp.	218	0.184564327
Nitrosomonas sp.	210	0.177791324
Beta Proteobacterium	198	0.16763182
Nitrospira sp.	186	0.157472315
Endosymbiont of *Syntrophobacter sp.*	178	0.150699313
Syntrophobacter sp.	177	0.149852687
Bos taurus	176	0.149006062
Alpha Proteobacterium	175	0.148159436
Conexibacter woesei	168	0.142233059
Bacillus licheniformis	148	0.125300552
Pirellula staleyi	147	0.124453927
Uncultured *Bacteroidetes*	137	0.115987673
uncultured *Rubrobacteridae*	136	0.115141048
Rhodococcus ruber	120	0.101595042
Opitutus sp.	115	0.097361915
uncultured *Actinomycete*	19	0.016085882

The analysis showed great microbial diversity in the reactor. The majority of the observed sequences were from bacteria of known origin. The *Bacillus subtilis* were the most dominant genus (44%) in the total microbial community. The *Geobacillus stearothermophilus* which presented 24% of total population was the second largest genus. Bacillus species presented 17% of the total population and were the third largest genus (Table 4-1). Some of the reasons that can explain the overabundance of bacillus species in the reactor are their ability to survive in most environmental conditions and their involvement in ammonia assimilation process. However, *Planctomycetes* in which anammox bacteria belong to presented a

considerable amount of 4% of the total microbial population. In addition, 1 % of uncultured ammonium oxidizing bacteria was also observed. This made it possible for anammox bacteria to be the fourth largest group of bacteria presenting a total of 5% in the reactor. About 4 % of the bacteria could not be classified to a known phylum of bacteria domain.

4.4 Conclusion

After sequencing the V3 and V4 regions of the 16s rRNA using next generation sequencing, a complex microbial community was identified. Although anammox bacteria were not the most dominant bacteria in the system, a significant amount (5%) of these bacteria was observed. These results demonstrate that anammox system was successfully operated but great improvements are required in order to obtain large anammox biomass for industrial scale.

5. General Conclusion

In this study, a process for the start-up and enrichment of anammox bacteria from wastewater treatment was developed and tested in laboratory batch reactors. Anammox biomass was successfully enriched from the sludge samples from sludge samples with very low source anammox bacteria. The anammox cultures were enriched from three sludge sources from treatment plants around Pretoria. Identification of anammox bacteria in batch reactors was done by PCR analysis. These results showed that anammox bacteria are ubiquitous in wastewater treatment plants in South Africa. The detected anammox bacteria belonged to the *Brocadia* species (*fulgida* and *anammoxidans*).

A sequencing batch reactor (SBR) was set up and operated for the removal of nitrogen compounds in wastewater. Samples of biomass from successfully enriched cultures were used as inoculum for the SBR. The systems worked effectively, achieving the nitrite removal rate of 97 %. Samples of biomass obtained from the SBR were analysed for identification of the main bacterial populations present in the reactor. Next generation sequencing (NGS) was used for identification of bacterial population. The reactor was found to be dominated by different bacillus species with abundance of 84%. Although the over-abundance of these bacterial species is not clear, it could be associated with the nature of the bacteria which is very resistant and able to survive in most environmental conditions. Additionally, their involvement in the nitrogen assimilation process could also explain their over-abundance in the reactor. However, a total of 5% anammox and planctomycetes bacteria was also seen in the reactor. *Nitrosomonas* species which are known ammonia oxidizers were also present in the reactor though in very small amounts (0.2%).

6. References

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